Finding the Right Partner: Science or ART?

Simona Polo[1,2,*] and Pier Paolo Di Fiore[1,2,3,*]
[1]IFOM, Istituto FIRC di Oncologia Molecolare, Via Adamello 16, 20139 Milano, Italy
[2]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Universita' di Milano, Via di Rudini, 20122 Milano, Italy
[3]Istituto Europeo di Oncologia, Via Ripamonti 435, 20139 Milano, Italy
*Correspondence: simona.polo@ifom-ieo-campus.it (S.P.), pierpaolo.difiore@ifom-ieo-campus.it (P.P.D.F.)
DOI 10.1016/j.cell.2008.10.032

In response to environmental cues, certain plasma membrane proteins are ubiquitinated, signaling their endocytosis and degradation. In the budding yeast, a single enzyme, Rsp5, is charged with this task. Lin et al. (2008) now identify an arrestin-related family of Rsp5 adaptor proteins called ARTs that confer specificity on the ubiquitination of plasma membrane proteins and contribute to the maintenance of the correct cell surface protein repertoire.

Hundreds of different proteins at the plasma membrane control interactions between the cell and its extracellular milieu, governing virtually every aspect of cellular homeostasis. Maintenance and rapid remodeling of the plasma membrane protein repertoire in response to environmental cues is critical for correct functioning of the cell. This is underscored by the fact that defects in these processes underlie many human diseases, including cancer and neurodegenerative disorders. One way to control the plasma membrane protein repertoire is through the process of endocytosis. Ubiquitination is an evolutionarily conserved mechanism for signaling that a protein (cargo) is to be endocytosed (Mukhopadhyay and Riezman, 2007) and is executed by ubiquitin ligases (E3s). In this issue, Lin et al. (2008) report the identification of a family of proteins—arrestin-related trafficking adaptors, or ARTs—that may mediate interactions between the ubiquitin ligase Rsp5 and protein cargoes during endocytosis in the budding yeast *Saccharomyces cerevisiae*. Their findings have far-reaching implications for understanding how the specificity of ubiquitination is determined and how the cell maintains the proper repertoire of proteins at the plasma membrane.

The yeast E3 Rsp5 (a homolog of mammalian Nedd4 family proteins) controls most trafficking-related ubiquitination events at the plasma membrane and at other biomembranes (Belgareh-Touze et al., 2008). Rsp5 recognizes, through its WW domain, proteins containing a PY motif, a sequence generally consisting of two adjacent prolines followed by any amino acid and then by a tyrosine (PPXY) (Belgareh-Touze et al., 2008). However, as most membrane proteins do not contain these motifs, how does Rsp5 mediate the ubiquitination of cargo destined for endocytosis? Lin et al. (2008) now describe a family of proteins called ARTs that display PY motifs and act as specific adaptors between plasma membrane cargo proteins and Rsp5. A few Rsp5 adaptors (Bul1/Bul2, Bsd2/Tre1/Tre2, Ear1/Ssh4) are known (Belgareh-Touze et al., 2008), but the discovery of a family of proteins putatively endowed with a specific adaptor property shows that the adaptor mechanism is more extensive than previously thought (although some caution is in order as Lin et al. only fully characterize ART1 and ART2 of the nine-membered family).

The predicted structures of seven of the nine ART proteins display an arrestin fold. This structural element is the hallmark of the arrestin family that includes β-arrestins, proteins that mediate the endocytosis of G protein-coupled receptors (Figure 1) (Alvarez, 2008; DeWire et al., 2007). Although the ARTs do not show strong sequence homology to arrestins, they all contain a short amino acid motif (termed by the authors as an "arrestin motif") that is highly conserved in members of the arrestin family. Most importantly, ART1 and ART2 share with β-arrestins the E3 adaptor function. In addition, all but one of the ARTs associate with Rsp5 in pulldown experiments, suggesting that these family members may also be endowed with the adaptor function. Thus, not only can interesting parallels be drawn between the mechanisms of ART- and β-arrestin-mediated endocytosis, but there may also be a general role for the arrestin superfamily in determining the specificity of ubiquitination (Figure 1).

How do the ARTs function? An obvious possibility is that they simply provide a physical link between Rsp5 and the cargo. Lin and colleagues showed that ART1 is required for endocytosis of the methionine transporter Mup1 and of the arginine transporter Can1. In the case of Mup1, they showed methionine-dependent interactions between ART1 and Mup1. They also demonstrated that ART1 relocates to the plasma membrane in response to environmental signals that cause the endocytosis and degradation of Can1. Can1 engineered to contain the PY motif of ART1 is constitutively targeted to the vacuole for degradation. Thus, ART1 may mediate the association of Rsp5 with cargo proteins resident in the plasma membrane through its PY motif. Although Lin et al. did not test whether ART1 is required to target Rsp5 to the plasma membrane, data from other studies suggest a role for adaptors in directing Rsp5/Nedd4 to biomembranes in both yeast and mammals (Belgareh-Touze et al., 2008). An alternative but not mutually exclusive possibility is that ARTs might control the catalytic activity of Rsp5. There is a precedent for this type of regulatory mechanism in mammals where the E3 Smurf2 (a Nedd4 family ligase) is catalytically regulated by the adaptor Smad7 (Wiesner et al., 2007). It remains to be established whether such a mechanism can be extended to the ARTs.

Lin and colleagues also unveil an additional layer of complexity in the regulation of ART-mediated endocytosis. They

demonstrate that ubiquitination of ART1 itself by Rsp5 is necessary for its endocytic function. How ubiquitination regulates ART1 remains to be determined, but Lin et al. do show that ubiquitination is required for the correct subcellular localization of ART1. That Rsp5 is responsible for the ubiquitination of both the adaptor and the cargo suggests that there is a feed-forward loop in ART-mediated regulation of endocytosis, which may be a general phenomenon, given that other Rsp5 adaptors and β-arrestin are also ubiquitinated by their partner ligases (Belgareh-Touze et al., 2008; Shenoy et al., 2008).

The most important feature of ARTs is that they may confer specificity on Rsp5 during cargo recognition. ART1, for instance, is required for endocytosis of three plasma membrane proteins (Can1, Mup1, and Lyp1) but not of several others (Fur4, Ftr1, Pdr5, and Ste2). But how do the ARTs specifically recognize their cargo? Interestingly, Lin et al. report that the lysine transporter Lyp1 requires either ART1 or ART2 for its endocytosis, depending on the type of stimulus. This indicates that the surfaces of adaptor or cargo proteins alone are unlikely to account for specificity. Instead, specificity may be mediated, at least in part, by regulation of the adaptor-cargo interaction. Although other mechanisms cannot be excluded, stimulus-specific posttranslational modifications of cargo proteins are the prime suspects for mediating this regulation. Indeed, several yeast and mammalian plasma membrane proteins need to be phosphorylated before they can be ubiquitinated and internalized (Belgareh-Touze et al., 2008). A new study reports that two ARTs (Ecm21 and Csr2, corresponding to ART2 and ART8, respectively) act as Rsp5 adaptors in the ubiquitination and endocytosis of the yeast metal transporter Smf1 and that phosphorylation of the cargo is required for Ecm21 binding (Nikko et al., 2008). Cargo recognition may also be finely tuned at multiple levels. ARTs could be promiscuous, with overlap in their cargoes. It is also possible that there is redundancy among Rsp5 adaptors. Although this was not investigated for ARTs, it is true for the Rsp5 adaptor pairs Tre1/Tre2 and Ear1/Ssh4 (Belgareh-Touze et al., 2008). Furthermore, cooperation between adaptors might be required, as demonstrated for the Rsp5 adaptors Bsd2 and Tre1/Tre2 (Belgareh-Touze et al., 2008). This possibility evokes an intriguing scenario whereby the combinatorial action of different adaptor sets is required to guide E3s to their specific cargoes.

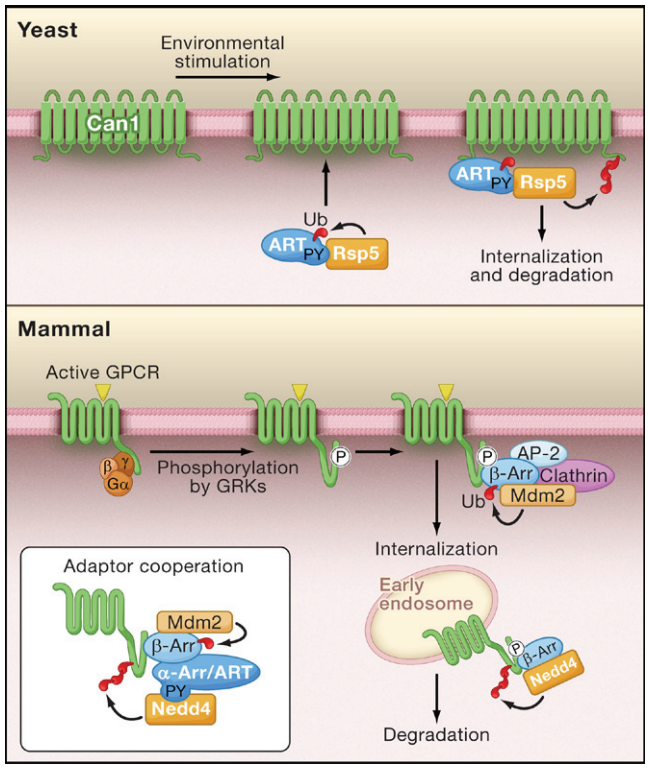

Figure 1. Cargo Endocytosis by ARTs and β-Arrestins

(Top) In yeast, arrestin-related trafficking adaptors (ARTs) and the E3 ubiquitin ligase Rsp5 are recruited to the plasma membrane in response to environmental stimuli that trigger the endocyotosis of proteins such as permeases and transporters (e.g., the arginine transporter Can1). Through their PY motif, ARTs bind to Rsp5 and mediate ubiquitination (red) of cargo (Can1). The ubiquitinated cargo is then internalized and degraded. ARTs are also ubiquitinated by Rsp5, an event required for endocytosis.

(Bottom) In mammals, activated G protein-coupled receptors (GPCRs) signal through heterotrimeric G proteins. This mechanism is terminated when GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs), leading to the recruitment of β-arrestin. β-arrestin binds to AP-2 and clathrin to enable the internalization of the GPCR cargo. Mdm2-mediated ubiquitination of β-arrestin is necessary for GPCR internalization. GPCRs can also be ubiquitinated, most likely by Nedd4 (the mammalian Rsp5 homolog), an event required for cargo degradation but not internalization (Shenoy et al., 2008). The inset shows a possible mechanism for cooperation between β-arrestin/Mdm2 and α-arrestin (the putative mammalian ART protein homolog)/Nedd4 that may result in the coordinated ubiquitination of β-arrestin and GPCRs.

The recent in silico identification of a mammalian family of orphan arrestins (α-arrestins, ARRDC in human) further suggests the possibility of adaptor cooperation (Alvarez, 2008). These α-arrestins may be the mammalian homologs of ARTs and also may represent E3 adaptors that regulate endocytosis of cargo proteins in mammalian cells. Interestingly, β-arrestins emerged later in evolution than α-arrestins and diverged to become a specialized version of the ancestral arrestin, able to interact with the endocytic machinery (a property missing in ARTs and α-arrestins; Figure 1) (Alvarez, 2008). Because α- and β-arrestins are widely coexpressed and in principle are capable of heterodimerization, they may function coordinately to finely tune both cargo recognition and recruitment of the endocytic machinery (Figure 1) (Alvarez, 2008). This potential for adaptors to work in combination may resolve the conundrum of the existence of considerably fewer E3 ligases than ubiquitinated substrates; there are only a few dozen E3s in yeast for over 1000 known substrates (Peng et al., 2003). Adaptor cooperation with E3 ligases would confer enough combinatorial capacity to cope with the large number of substrates. In principle, 50 adaptors could give rise to more than 1200 distinct binary combinations. Whether this speculative scenario is true in vivo is worthy of further study.

ACKNOWLEDGMENTS

S.P. is supported by the Italian Association for Cancer Research (AIRC) and the Association for International Cancer Research (AICR). P.P.D.F. is supported by the AIRC, the European Community (VI Framework), the Ferrari Foundation, the Monzino Foundation, and the CARIPLO Foundation.

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Pol V Transcribes to Silence

Lucia Daxinger,[1] Tatsuo Kanno,[1] and Marjori Matzke[1,*]

[1]Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Dr. Bohr-Gasse 3, A-1030 Vienna, Austria

*Correspondence: marjori.matzke@gmi.oeaw.ac.at

DOI 10.1016/j.cell.2008.10.027

In most cases, the functions of long noncoding RNAs remain uncertain. Working in the model plant *Arabidopsis*, Wierzbicki et al. (2008) provide evidence that transcription of intergenic noncoding regions by RNA polymerase V promotes heterochromatin formation and silencing of nearby genes.

A group of unusual RNA polymerase subunits has intrigued plant biologists ever since their discovery during the initial analysis of the *Arabidopsis* genome. In addition to DNA-dependent RNA polymerases I, II, and III, flowering plants have two extra RNA polymerases termed Pol IV and Pol V. Genetic experiments have implicated Pol IV and Pol V in chromatin-based gene silencing mediated by small RNAs (Pikaard et al., 2008), and current models suggest that the two polymerase complexes act at different steps of the silencing pathway: Pol IV produces and amplifies the small RNA trigger, whereas Pol V functions downstream to facilitate de novo DNA methylation at the site targeted by the small RNA. Although Pol IV is involved in the production of 24 nucleotide (nt) "heterochromatic" small-interfering RNAs (siRNAs) (Mosher et al., 2008), the function of Pol V in gene silencing is unclear. For instance, it is unknown whether Pol V transcribes extensively or simply opens chromatin at the siRNA-targeted site to expose DNA to cytosine methyltransferases (Kanno et al., 2005).

A study published in this issue of *Cell* (Wierzbicki et al., 2008) provides the first evidence of transcripts generated by Pol V and documents their role in chromatin-based gene silencing. The newly discovered Pol V transcripts originate from intergenic noncoding regions and facilitate siRNA-directed epigenetic modifications that impede transcription of overlapping and neighboring transposons and genes by Pol II and Pol III. The findings nicely illustrate how plants have diversified their transcriptional machinery to include specialized RNA polymerases that participate in the formation of repressed chromatin for gene silencing.

Prior work in fission yeast has shown that RNAi-mediated formation of heterochromatin depends, paradoxically, on Pol II transcription of the target sequence. The Pol II-generated noncoding RNAs have a dual function in heterochromatin assembly, serving both as precursors for siRNAs and as scaffolds that interact with siRNAs to recruit chromatin-modifying factors. A JmjC domain-containing protein, Epe1, enables Pol II transcription of heterochromatic repeats by counteracting repressed chromatin (Zofall and Grewal, 2006). Given the precedent for Pol II-generated noncoding RNAs in siRNA-mediated heterochromatin formation in fission yeast, Wierzbicki and coworkers sought to determine whether Pol IV and/or Pol V transcribe noncoding RNAs in plants.

The authors first suspected Pol V transcription from their inspection of the heterochromatic knob on *Arabidopsis* chromosome 4. Although this region is hypermethylated and encodes numerous siRNAs, certain areas are devoid of detectable RNA transcripts on DNA tiling arrays. Working on the hypothesis that low abundance transcripts might provide precursors for siRNAs, they used RT-PCR to look for intergenic noncoding RNAs that disappear in plants lacking Pol IV or Pol V. Of 14 regions examined, 6 gave rise to RNAs that were lost only in plants deficient in Pol V. Four intergenic noncoding transcripts were derived from transposon and repeat-rich regions, whereas two originated from gene-rich portions of the genome, indicating that Pol V transcribes in both